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Up-regulation of SIBLING proteins and correlation with cognate MMP expression in oral cancer

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Summary Various combinations of the SIBLING family of proteins have been found to be up-regulated in many human cancers and have been linked to different stages of tumor progression, including metastasis. Bone sialoprotein (BSP), osteopontin (OPN) and dentin matrix protein 1 (DMP1) specifically bind and activate MMP-2, MMP-3, and MMP-9, respectively. These proteases have also been shown to play important roles in oral squamous cell carcinoma (OSCC) invasion and metastasis. However, with the exception of OPN, there are no reports on the expression of the family of five SIBLING proteins in OSCC. This study examines the expression patterns of the SIBLING family (and MMP partners when known) in OSCC, correlating expression to outcome variables. Archived paraffin sections of 87 cases of primary OSCC were screened by immunohistochemistry for the SIBLINGs and their MMP partners. Three SIBLINGs (BSP, DSPP, and OPN), were expressed in OSCC, while DMP1 and MEPE expression were never observed. Furthermore, BSP and OPN were always expressed with their known MMP partners, MMP-2 and MMP-3,

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respectively. Poorly differentiated tumors exhibited reduced or no immunoreactivity for BSP and OPN but increased immunoreactivity for DSPP. Seventy eight (90%) cases were positive for BSP and DSPP, while 79 cases (91%) were positive for OPN. Overall, 91% of the cases were positive for at least one SIBLING. There were no correlations between SIBLING expression and tumor size (''T''; of the Union Internationale Contre le Cancer [UICC]-TNM classification for OSCC), and between SIBLING expression and lymph node spread for the T1/T2 tumors. The levels of DSPP expression for floor of mouth and retromolar region tumors were higher than for tongue tumors. Statistically significant correlations were, however, found between the expression levels of BSP and MMP-2 ($p < 0.0001$), BSP and MMP-3 ($p < 0.0001$), and OPN and MMP-3 ($p < 0.0024$). We conclude that BSP, DSPP, and OPN are highly up-regulated in OSCC. While the production of these SIBLINGs is independent of T, they correlate with oral location of tumor, cognate MMP expression, and for DSPP, the degree of tumor differentiation.

Introduction

Oral cancer is the sixth most common cancer in the world and the incidence of new cases indicates a continuing rise in developing countries.[1,2] About 30,000 new cases of oral and oropharyngeal cancers are diagnosed annually in the United States with about 7,500 resultant deaths.[3,4] For the last four decades, the mortality rate from oral cancer has remained high (~50%), in spite of new treatment modalities.[2] Over 90% of oral malignancies are histologically characterized as oral squamous cell carcinomas (OSCC).[3] Most OSCC patients die as a result of local and regional spread of the disease and not of distant organ metastasis.[2]

On the basis of their common genetic and structural features as well as interactions with other acknowledged family groups such as integrins and matrix metalloproteinases (MMPs), bone sialoprotein (BSP), osteopontin (OPN), dentin sialophosphoprotein (DSPP), dentin matrix protein 1 (DMP-1), and matrix extracellular phosphoglycoprotein (MEPE) have been proposed to constitute a gene family called the SIBLINGs (Small Integrin-Binding LIgand, N-linked Glycoproteins).[5,6] The SIBLINGs are encoded by a tandem cluster of genes within a ~375,000 basepair region of human chromosome 4.[5] The four acidic SIBLING proteins, BSP, OPN, DMP1, DSPP, were discovered many years ago embedded in the mineralized matrices of bone and teeth by many laboratories.[6,7] The basic protein, MEPE, was discovered more recently in association with tumors that cause phosphate wasting, but is also expressed in the skeleton.[8] With the exception of OPN (previously reported to be expressed in non-mineralizing tissues such as the kidney, lactating mammary gland, and certain immune cells,[9–11]) the SIBLINGs were generally thought to be limited to bones and teeth in normal adult tissues. Results of recent studies however indicate that all five members of the SIBLING family are expressed in the ductal epithelial cells of normal adult salivary glands[12] and kidney.[13]

Over the last few years, the up-regulation of the SIBLING family members has been reported for a number of cancers.[14–28] For example, OPN and BSP have been linked with different stages of tumor progression: cell growth; adhesion; migration and/or metastasis,[28] and published reports indicate that various combinations of SIBLINGs are up-regulated in breast,[14,15] prostate,[16,17] lung,[18,19] and colon cancers.[26] High BSP and OPN expression in breast and prostate primary tumors are significantly associated with poor prognosis and the development of bone metastases in these diseases.[17,21–25] More recently, Chaplet and colleagues found that DSPP is up-regulated in prostate cancer.[27] Cancers of these organs however differ from oral cancer in two significant respects. First, cancers of the breast, prostate, lung, and colon are essentially adenocarcinomas while oral cancer is predominantly squamous cell carcinoma. Second, cancers of the breast, prostate, lung, and colon are characterized by their high tendency to metastasize to distant sites early in the course of the disease, while in OSCC distant metastasis beyond regional cervical lymph nodes, if it occurs at all, remains a late event.

Recent reports also document the specific partnering and co-localization of MMP-2, MMP-3, and MMP-9 with three members of the SIBLING family: BSP; OPN; and DMP1, respectively, both *in vitro* and *in vivo*.[12,13,29] There are also numerous reports documenting the expression of MMP-2, MMP-3, and MMP-9 in OSCC tissues and cell lines, but except for OPN,[30–32] there are no reports on the expression of the other SIBLINGs in human OSCC. Sasaguri et al. demonstrated the presence of BSP mRNA and protein in the chemically-induced hamster buccal-pouch OSCC model system.[33]

We have screened archived paraffin sections of surgical resections from 87 patients with primary OSCC for the presence of all five SIBLINGs and the three known MMP partners,[12,13,29] using immunohistochemistry. Our objectives were to determine which of the SIBLINGs and their MMP partners are expressed in OSCC, and the extent to which each expression is related to tumor size (T). In addition, we sought to examine the extent to which SIBLING-MMP expression in the T1/T2 OSCC relate to histologic differentiation, presence or absence of regional lymph node spread, and to other notable variables associated with outcome and prognosis in oral cancer. This is with a view to examining whether such expression provides new opportunities for predicting OSCC aggressiveness and prognosis.

We have limited our analysis of the relationship between SIBLING/MMP expression and prognostic outcomes in OSCC to the small (T1/T2) tumors because of their unpredictable course and outcome. While the large (T3 and T4) tumors, often presenting with preoperative N-positive neck disease, create no controversy as to the treatment approach required, considerable doubt and debate on the best management approach to the T1/T2 OSCC linger. For example, although OSCC patients with T1/T2 lesions often present with clinically negative (N0) nodes, these patients also have

a high risk (20—44%) of occult node metastasis,[38] providing postoperative histologic node examination as the only avenue of proof for the presence of regional node disease. This unpredictable behavior of the T1/T2 lesions continues to underscore the urgent need for reliable parameters for predicting node metastasis in T1/T2 tumors.

Methods

Case selection

The required Institutional Review Board (IRB) approvals for study on human tissues were obtained from the National Institute of Health, the University of Maryland, and the Medical College of Georgia. A total of 87 cases of surgically resected primary OSCC retained in the Department of Oral and Maxillofacial Pathology, School of Dentistry, University of Maryland, Baltimore were selected for this study based on the following criteria:

Inclusion criteria:

1. Resected primary tumors were located in the oral cavity and/or oropharynx;
2. Diagnosis of OSCC was confirmed independently by two oral pathologists;
3. All cases of T1/T2 have adequate clinical and follow-up data, including histologic post-operative neck node status where applicable.

Exclusion criteria: The following were the basis for exclusion:

1. Presence of secondary primary tumors;
2. Presence of metastases to the oral cavity from other sites;
3. Concurrent diagnosis of neoplasms of other regions;
4. A history of previous cancers of other organs or sites at the time of OSCC diagnosis and treatment.

The staging of selected cases was based on the Union Internationale Contre le Cancer (UICC)-TNM (Tumor-Size, Node, Metastases) tumor classification.[34] T defines the tumor size at the primary site, N the presence or absence of regional (submandibular and cervical) node spread, and M the presence or absence of distant metastasis at the time of the initial diagnosis of OSCC. Patients' clinical records indicated that 27 cases were classified as T1/T2 tumors (T1: $\leqslant$2 cm; T2: >2 cm but <4 cm in greatest dimension), 30 as T3 (T3: >4cm in greatest dimension), and 30 as T4 tumors (T4: tumor invades adjacent structures). The 87 cases of primary OSCCs satisfying the inclusion criteria were randomly selected. The racial/ethnic/gender make-up of selected cases reflected the same racial/ethnic proportion of the population of the Baltimore area.

Histopathologic analysis

Representative sections of all tumors were reviewed by two oral and maxillofacial pathologists (NN and KUO) who were blinded to the lymph node status of each case. The tumors were classified based upon a modification of the system proposed by Anneroth et al.[35] Six parameters were evaluated: (a) keratinization; (b) nuclear pleomorphism; (c) number of mitoses per high power field; (d) pattern of invasion; (e) stage of invasion; and (f) lymphocytic infiltration, paying particular attention to the most dysplastic area of the invasive front.[36] Each of the 6 parameters were scored for each tumor using a 4-point rating scale, with a score of 1 corresponding to a more differentiated presentation (e.g. abundant keratinization), and a score of 4 corresponding to the least differentiated presentation (e.g. absence of keratinization). A composite score was also calculated for each tumor as the sum of the individual scores in this classification system. Additionally, the tumor thickness was measured in millimeters microscopically for each tumor, as described by Moore et al.[37] Briefly this involves measuring from the surface of the epithelium to the deepest invading tumor island or cell, using a reconstructed line excluding any exophytic component of the tumor and including the thickness of epithelium that is lost due to ulceration.

Immunohistochemistry

All the SIBLING antibodies used for this study were produced in the laboratory of one of the authors (LWF) and have been previously published.[12,13] The SIBLING monoclonal antibodies used were LFMb-25 for BSP, LFMb-14 for OPN, LFMb-21 for DSPP, LFMb-31 for DMP1, and LFMb-33 for MEPE.[12,13] Their polyclonal counterparts LF-84 (affinity purified), LF-123, LF-151, LF-148 (affinity purified), and LF-155 (affinity purified), respectively, were used to validate corresponding monoclonal antibody results.[12,13] Human MMP-2, MMP-3 and MMP-9 were detected using rabbit antibodies generated against MMP-specific synthetic peptides conjugated to keyhole limpet hemocyanin protein through the cysteine in each peptide. (MMP-2, LF-183: ENQSLKSVKFGSIKSDWLGC; MMP-3, LF-182: EPGFPKQIAEDFPGIDSKIDAC; and MMP-9, LF-184: RSELNQVDQVGYVTYDILQCPED). In each case, the antibody was affinity purified using the same peptides conjugated to activated agarose beads. Each antiserum showed no cross-reactivity with the other two authentic human MMPs on ELISA assay.

Immunostaining was carried out using the Zymed ST5050 automated system (Zymed Lab Inc., San Francisco, CA) as previously described.[12,13] In brief, 4 μm paraffin sections were manually dewaxed in three xylene washes (5—10 min each) and then rehydrated through graded ethanol (100%, 95%, and 75%) and deionized water. Endogenous peroxidase activity was destroyed by treating the sections for 30 min with 3% hydrogen peroxide in methanol. Sections were thereafter washed 3 times in phosphate-buffered saline (PBS) for at least 5 min each and covered with PBS + 0.05% Tween-20 (PBS-T) before loading the slides onto the preprogrammed ST5050 automated immunohistochemistry machine.

Sections were incubated for 1 h with appropriate SIBLING antibody diluted in 10% normal goat serum in PBS. The sections then underwent a 4×1 min wash cycle with PBS-T before incubation with SuperPicTure Polymer horseradish-peroxidase (HRP)-conjugated broad-spectrum secondary antibody (#87-8963, Zymed Lab. Inc., San Fransisco, CA,

USA) for 10 min. Thereafter, sections were passed through another wash cycle and were developed with AEC (amino ethyl carbazol) Single Solution chromogen (#00-1122, Zymed Lab. Inc., San Francisco, CA, USA) for 2 min. Counterstain with Mayer's hematoxylin for 10—20 s was carried out manually before applying an overlay of Clearmount (Zymed Lab. Inc., San Francisco, CA) glaze. After drying, slides were coverslipped with Histomount (Zymed Lab. Inc., San Francisco, CA, USA). All steps were performed at room temperature. Negative controls included the substitution of primary antibody with non-immune rabbit serum or mouse IgG control (#08-6599, Zymed Lab. Inc., San Francisco, CA, USA). Photographic images of representative reproducible experimental results for each selected case were captured using the Axioplan 2 Universal microscope equipped with an Axiovision digital camera and Axiovision program (Carl Zeiss Gmbh, Jena, Germany).

Scoring of immunohistochemistry results

Two oral pathologists (KUO and NN) reviewed the post-surgical diagnoses of all selected cases independently to confirm the diagnosis of OSCC. Clinicopathological details of patients with T1/T2 tumors were extracted from the patients' charts and retained by one of the investigators (GW). The pathologist (KUO) scoring the immunohistochemistry results was blinded to the clinicopathological details of all cases until after the completion of the evaluation and scoring. Immunohistochemistry staining for protein was evaluated semiquantitatively. Scoring was as negative (0, not detectable), 1 (detectable but <50% of tumor cells), 2 (present in >50% but <75% of tumor cells), and 3 (widely and highly expressed). Any extra-epithelial staining was also documented. Selective *in situ* hybridization for mRNA of each SIBLING was carried out to verify immunohistochemistry results.

Statistical analysis

To examine whether the levels of SIBLING expression correlate with tumor sizes (T1/T2, T3, or T4), a chi-square test was employed. Similarly, the chi-square test was used to examine differences in SIBLING expression levels among degrees of histologic differentiation (well-, moderate-, poorly-differentiated). When the assumptions for the chi-square test were not met (i.e., that the expected value in each cell of the table was greater than 5) then a Fisher's Exact test was employed.

Clinical data for the 27 cases of T1/T2 tumors were analyzed. Chi-square tests (or Fisher's Exact tests) and Kruskal—Wallis tests were used to examine potential differences in various demographic variables (sex, race, age), health status (smoking and alcohol consumption), location of the tumor in the mouth, histology, regional lymph node spread, various inflammatory responses, and other clinical variables. Non-parametric tests were used for each SIBLING due to the low sample size and due to the ordinal nature of some of the variables. All statistical analyses were performed using SAS 9.1.3 (SAS/STAT software, Version 9.1.3 of the SAS System for Windows, SAS Institute Inc., Cary, NC, USA.), and statistical significance was assessed using an alpha level of 0.05.

Results

Immunolocalization of SIBLINGs in primary OSCC resections

First, the expression of each SIBLING was determined by immunohistochemistry in successive paraffin slides of each OSCC block and verified by *in situ* hybridization in specific cases. Results indicated that one or more of the three SIBLINGs (BSP, DSPP, and OPN) were expressed in OSCC, while DMP1 and MEPE expression were absent in all 87 cases (illustrative examples are shown in Figure 1). As shown in Figure 1A immunoreactivity for BSP was evident within the cytoplasm and the perinuclear perimeters of proliferating tumor cells with significant intensity in well-differentiated tumor foci. Similarly, immunoreactivity for OPN (Fig. 1B) was in both the cytoplasm of malignant epithelial cells and in reactive immune cells infiltrating the connective tissue stroma (Fig. 1B arrow). DSPP immunoreactivity was confined to the cytoplasm and perinuclear perimeter of malignant epithelial cells alone (Fig. 1C). The connective tissue stroma itself in all cases showed negative immunoreactivity for all five SIBLINGs. Figure 1D is a representative result showing the non-immune mouse IgG negative controls for the SIBLINGs within tumor cells and associated connective tissue elements.

BSP and OPN co-localization with MMP-2 and MMP-3, respectively

We recently reported that three of the SIBLINGs: BSP; DMP1; and OPN are co-localized with specific MMPs in some normal duct epithelial tissues.[12,13] As shown in Figure 2, MMP-2 (2A) and BSP (2B) were expressed in the cytoplasm of tumor cells. So also were MMP-3 (2C) and OPN (2D), suggesting that the SIBLING-MMP pairing previously observed in some normal duct epithelia may also occur in disease conditions. Furthermore, although poorly differentiated tumor cells retained their expression of MMP-2, MMP-3, and MMP-9 (Fig. 3B, illustrative of punctate MMP-2 expression in poorly differentiated area of tumor), they consistently and completely lacked BSP immunoreactivity (3A; arrows indicate positively immunoreactive benign salivary duct epithelium caught up in tumor and serves as built-in positive control). Similarly, there was significant reduction or no immunoreactivity for OPN (3C) among poorly differentiated tumor cells. In contrast, DSPP was significantly upregulated in the cytoplasm of poorly differentiated OSCCs (3D).

Relationship between level of SIBLING expression in primary OSCC and tumor size (T)

Table 1 summarizes the semiquantitative scores for the immunoreactivity of each SIBLING among the T1/T2, T3, and T4 OSCCs in this study.

T1/T2 tumors: The levels of BSP expression in two of the 27 (7.4%) T1/T2 tumors were scored as 1 (<50% immunoreactivity; as represented in Fig. 1A) when assessed under a high-power (40×) light microscopic field. Eleven (41%) of T1/T2 tumors were assigned a semiquantitative score of 2 (>50% but <75%), and another 11 (41%) scored as 3 (>75%

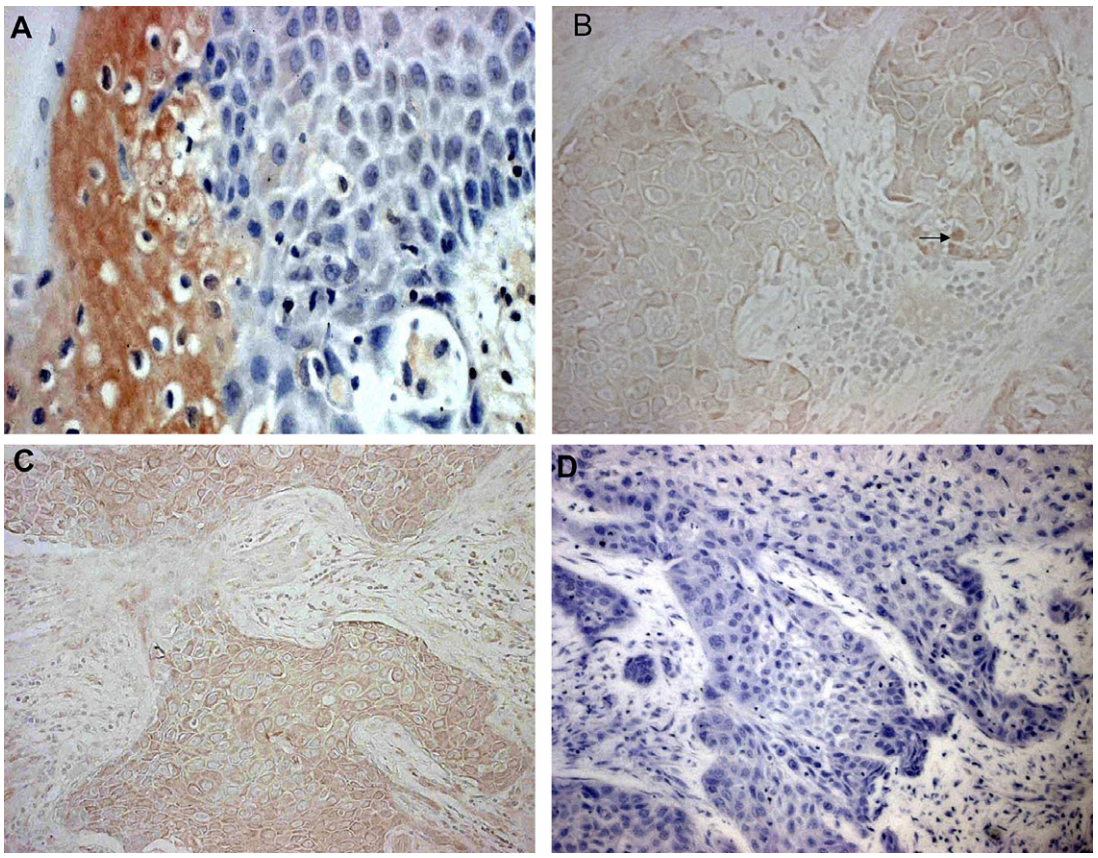

Figure 1 SIBLING immunolocalization in OSCC using representative semiquantitative analysis (reddish-brown = AEC; blue = hematoxylin counterstain) in paraffin sections. BSP expression in tumor scored as 1 for less than 50% tumor cell immunoreactivity (A) with BSP monoclonal antibody LFMb-25. Note the cytoplasmic staining of epithelial tumor cells, and the absence of staining in the connective tissue. OPN expression scored as *2* for more than 50% but less than 75% tumor cell immunoreactivity (B) with OPN monoclonal antibody LFMb-14. immunoreactivity is also cytoplasmic with occasional reactivity in immune cells (B; arrow). DSPP expression scored as *3* for over 75% tumor cell immunoreactivity (C) with DSPP monoclonal antibody LFMb-21. Representative pre-immune negative control (D) showed expression scored as 0 for no immunoreactivity.

of immunoreactivity) for BSP. Three (11%) were scored as 0 because they did not express BSP. With respect to DSPP, None of the T1/T2 tumors was scored as 1, while 11 (41%) were scored as 2 (>50% but <75% immunoreactivity as represented in Fig. 1C), and 13 scored as 3 for DSPP. Three (11%) tumors were scored as 0 for DSPP immunoreactivity. OPN immunoreactivity was scored as 1 for two (7.4%) of the T1/T2 tumors, while 14 (52%) were scored as 2 on the semiquantitative scale. Nine (33%) other T1/T2 tumors were scored 3 for OPN immunoreactivity (as represented in Fig. 1B). Two of the T1/T2 tumor (7.4%) failed to show immunoreactivity for OPN.

T3 tumors: Of the 30 T3 tumors, four (13.3%) were scored as 1, 13 (43.3%) scored as 2, and nine (30%) scored as 3 for BSP immunoreactivity. Four (13.3%) of the T3 lesions did not express BSP. With respect to DSPP immunoreactivity, four (13.3%) of T3 tumors were scored as 1, and another 15 (50%) scored as 2. Seven of the T3 tumors (23.3%) were scored as 3, and four others as 0 (no immunoreactivity) for DSPP. OPN immunoreactivity was scored as 1 for four (13.3%) T3 tumors, while 11 (37%) tumors were scored as 2 and another 11 (37%) as 3 for OPN immunoreactivity. Four of the T3 lesions were scored 0 (no immunoreactivity) for OPN.

T4 tumors: With respect to the 30 T4 tumors, four (13.3%) showed poor expression for BSP and semiquantitatively scored as 1, while 13 (43.3%) showed good expression for BSP scored as 2. Eleven T4 tumors (37%) showed good expression for BSP with a score of 3. Two (6.7%) were negative (score of 0) for BSP among the T4 lesions. DSPP immunoreactivity was poor (score of 1) in two (6.7%) of the T4 lesions, score of 2 in 13 (43.3%), and score of 3 in another 13 (43.3%) of the T4 tumors. Two (6.7%) of the T4 tumors were negative (score of 0) for DSPP. OPN expression in two (6.7%) of the T4 tumors received a score of 1, while 17 (57%) T4 tumors reacted much more positively (score of 2). Nine, T4 tumors (30%) showed diffuse immunoreactivity (score of 3), while two (6.7%) were negative (score of 0) for OPN.

Overall (Table 1), the levels of expression of BSP (Fisher's Exact $p = 0.9498$), DSPP (Fisher's Exact $p = 0.2873$), and OPN (Fisher's Exact $p = 0.7972$) were not different for the T component of the TNM classification. Thus level of expression of all three SIBLINGs in resected primary OSCC

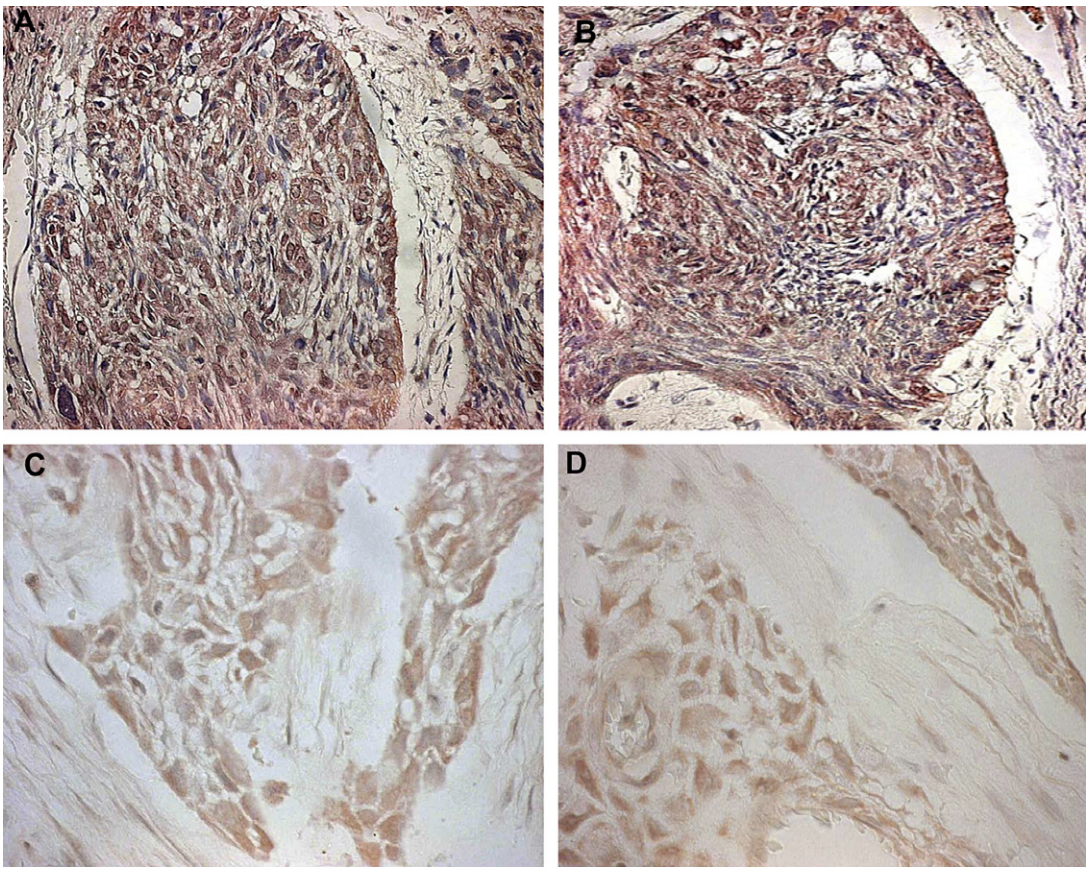

Figure 2 SIBLING-MMP expression shown in sections of OSCC. MMP-2 immunoreactivity with LF-183 in tumor cells within tumor island (A) was similar to BSP immunoreactivity with LFMb-25 in the cytoplasm of tumor cells (B). Similarly, MMP-3 immunoreactivity with LF-182 (C) and OPN immunoreactivity with LFMB-14 (D) were within the cytoplasm of tumor cells. MMP expression (A, C) was similarly confined to the cytoplasm of tumor cells but with occasional connective tissue foci of staining.

was not dependent on the T-status of primary tumor at the time of initial diagnosis.

Relationship between SIBLING expression levels of T1/T2 tumors and clinicopathologic variables

Tables 2—4 show the relationship of the various clinicopathologic variables to SIBLING expression levels (by scores 0—3) of BSP (Table 2), DSPP (Table 3), and OPN (Table 4) for the T1/T2 tumors. Analysis showed that the expression level of BSP (Fisher's Exact $p = 0.8575$), DSPP (Fisher's Exact $p = 0.5463$), and OPN (Fisher's Exact $p = 0.8942$) among moderate and well differentiated tumors was not statistically significant indicating that, within the spectrum of well- and moderately-differentiated OSCC, SIBLING expression was not a function of the degree of histological differentiation. Similarly, there were no statistically significant differences between SIBLING expression and sex, race, or the presence/absence of regional lymph node spread, pattern of invasion, or stage of invasion among the T1/T2 lesion.

However, a statistically significant difference was found between the level of DSPP expression and the location of primary OSCC. Tumors with scores of 2 or 3 for DSPP (Table 3) were more likely to be located at the floor of mouth (FOM) than the tongue. Also the lone retromolar pad tumor was scored as 3 for DSPP. With respect to the continuous measures, there were no statistically significant differences in the medians between SIBLING expression levels and age, depth of invasion, alcohol consumption, smoking consumption, keratin profile, lymph infiltration, nuclear polymorphisms, number of mitoses, pattern of invasion, or stage of invasion. Thus, the level of BSP, DSPP, and OPN expression in the primary tumor does not change significantly with any of the variables enumerated above.

SIBLING-MMP partners

For BSP and OPN, statistically significant differences in the median MMP-2 and MMP-3 levels were detected (Tables 2, 4). For BSP and its partner MMP-2 (Kruskal—Wallis chi-square = 15.0837, df = 3, $p = 0.0017$; Table 2), score of 3 had a significantly higher MMP-2 median than scores of 0 and 1. For BSP and MMP-3 (Kruskal—Wallis chi-square = 16.8922, df = 3, 0.0007; Table 2), scores of 2 and 3 also had significantly higher MMP-3 medians than scores of 0 and 1. For OPN and MMP-2 (Kruskal—Wallis chi-square = 11.3067, df = 3, $p = 0.0102$; Table 4), score of 3 had a significantly higher MMP-2 median than score 0, and score 2 had significantly higher medians than scores of 0 and 1. For OPN and its partner MMP-3 (Kruskal—Wallis chi-square = 10.9141, df = 3, 0.0122; Table 4), scores of 2 and 3 had significantly higher MMP-3

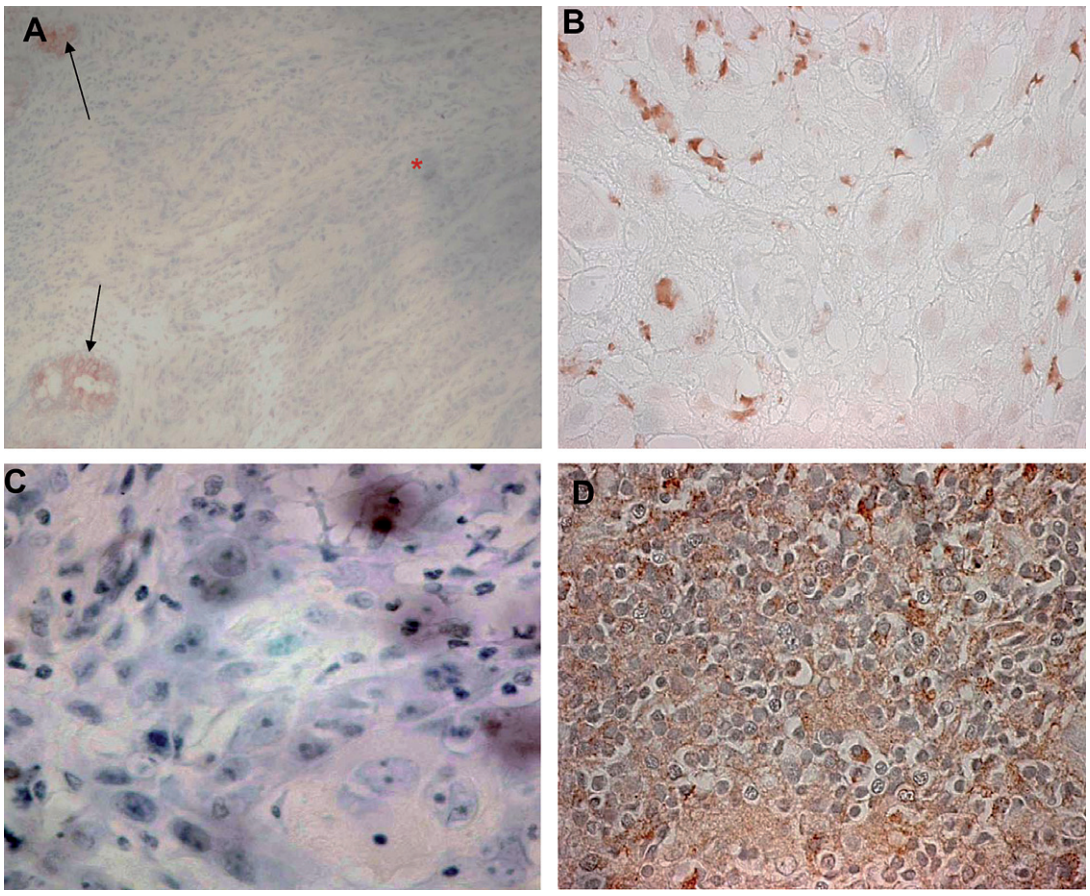

Figure 3 SIBLING-MMP expression in poorly differentiated OSCCs. Although MMP-2 immunoreactivity was retained among some poorly differentiated tumor cells (B), BSP immunoreactivity was completely negative (A) in poorly differentiated tumor areas (asterisk showing pleomorphic cells in advancing tumor front; arrows show positive immunoreactivity to BSP in normal salivary gland ducts that are caught up by invading tumor front. These serve as built-in positive controls). There was a significant reduction or complete absence of OPN immunoreactivity (C) in poorly differentiated tumors. On the other hand DSPP expression was significantly upregulated in poorly differentiated/anaplastic tumor areas (D). The phenotype of poorly differentiated/anaplastic tumor areas was confirmed by keratin stain (not shown).

Table 1 Semiquantitative scores for SIBLING immunoreactivity in primary OSCC[a]

	Total $n = 87$												Total # positive[a]	% Positive
	T1/T2 ($n = 27$)				T3 ($n = 30$)				T4 ($n = 30$)					
Scored as	0	1	2	3	0	1	2	3	0	1	2	3		
# Expressing BSP	3	2	11	11	4	4	13	9	2	4	13	11	78	90
# Expressing DMP1	27	0	0	0	30	0	0	0	30	0	0	0	0	0
# Expressing DSPP	3	0	11	13	4	4	15	7	2	2	13	13	78	90
# Expressing OPN	2	2	14	9	4	4	11	11	2	2	17	9	79	91
# Expressing MEPE	27	0	0	0	30	0	0	0	30	0	0	0	0	0

T = tumor size in the TNM system of tumor staging.
[a] Fisher's Exact p-value (BSP = 0.9498, DSPP = 0.2873, OPN = 0.7972) were *not different* due to T-status of tumor.

medians than score of 0. Statistically significant Spearman Rank correlations were found between OPN and MMP-3 levels ($p < 0.0001$), between BSP and MMP-2 ($p < 0.0001$), and between BSP and MMP-3 levels ($p < 0.0024$; Table 5). However, no correlations were found for BSP, DSPP, and OPN expression levels and MMP-9 (Table 5).

Discussion

In this study, the expression of the SIBLINGs in resected primary OSCC was studied using immunohistochemistry. Results showed that BSP, OPN, and DSPP were expressed in OSCC of all T-stages, while there was a complete lack of

Table 2 Relationship between BSP expression levels of T1/T2 tumors and clinical variables

Variable	0	1	2	3	*p*-Value
Sex[a]					0.9323
Male	4 (66.7)	1 (50.0)	6 (66.7)	7 (77.8)	
Female	2 (33.3)	1 (50.0)	3 (33.3)	2 (22.2)	
Race[a]					0.7508
Black	1 (16.7)	0 (0.0)	0 (0.0)	1 (11.1)	
Caucasian	5 (83.3)	2 (100.0)	9 (100.0)	8 (88.9)	
Tumor location[a]					0.4314
FOM	2 (33.3)	0 (0.0)	6 (66.7)	5 (55.6)	
Retromolar pad	0 (0.0)	0 (0.0)	0 (0.0)	1 (11.1)	
Tongue	4 (66.7)	2 (100.0)	3 (33.3)	3 (33.3)	
Histology[a]					0.2208
Poor	4 (25.0)	0 (0.0)	0 (0.0)	0 (0.0)	
Moderate	2 (50.0)	2 (100.0)	5 (71.4)	3 (33.3)	
Well	1 (25.0)	0 (0.0)	2 (28.6)	6 (66.7)	
Metastasis[a]					0.8161
Yes	1 (16.7)	1 (50.0)	3 (33.3)	3 (33.3)	
No	5 (83.3)	1 (50.0)	6 (66.7)	6 (66.7)	
Keratin[a,b]					0.0752
1	5 (83.3)	2 (100.0)	5 (55.6)	7 (77.8)	
2	0 (0.0)	0 (0.0)	4 (44.4)	0 (0.0)	
3	0 (0.0)	0 (0.0)	0 (0.0)	2 (22.2)	
4	1 (6.7)	0 (0.0)	0 (0.0)	0 (0.0)	
Pattern of invasion[a,b]					0.4420
1	1 (16.7)	1 (50.0)	2 (22.2)	1 (11.1)	
2	2 (33.3)	0 (0.0)	4 (44.4)	6 (66.7)	
3	1 (16.7)	0 (0.0)	3 (33.3)	1 (11.1)	
4	2 (33.3)	1 (50.0)	0 (0.0)	1 (11.1)	
Stage of invasion[a,b]					0.8848
1	1 (16.7)	0 (0.0)	2 (22.2)	1 (11.1)	
2	4 (66.7)	1 (50.0)	5 (55.6)	7 (77.8)	
3	1 (16.7)	1 (50.0)	2 (22.2)	1 (11.1)	
Age[c]	58.5 (45.0, 82.0)	68.5 (58.0, 79.0)	66.0 (49.0, 74.0)	65.0 (56.0, 86.0)	0.6041
Depth of invasion [c]	1.8 (0.7, 2.7)	4.3 (1.6, 7.0)	2.6 (0.5, 6.0)	2.0 (0.5, 4.5)	0.5289
ETOH[c]	0.0 (0.0, 7.0)	0.0 (0.0, 0.0)	5.0 (0.0, 10.0)	6.0 (0.0, 11.0)	0.3375
Smoking[c]	54.0 (0.0, 120.0)	382.5 (45.0, 720.0)	50.0 (0.0, 120.0)	46.0 (0.0, 240.0)	0.7708
Lymph infiltration[c,b]	2.0 (1.0, 4.0)	3.0 (3.0, 3.0)	3.0 (2.0, 3.0)	2.0 (1.0, 4.0)	0.6192
MMP-2[c]	1.5 (0.0, 3.0)	1.0 (1.0, 1.0)	2.0 (2.0, 3.0)	3.0 (2.0, 3.0)	0.0017
MMP-3[c]	1.0 (0.0, 2.0)	0.5 (0.0, 1.0)	3.0 (2.0, 3.0)	3.0 (2.0, 3.0)	0.0007
MMP-9[c]	0.0 (0.0, 2.0)	0.5 (0.0, 1.0)	0.0 (0.0, 2.0)	0.0 (0.0, 1.0)	0.6138
Nuclear polymorphism[c,b]	2.0 (1.0, 3.0)	2.0 (1.0, 3.0)	2.0 (1.0, 4.0)	1.0 (1.0, 4.0)	0.9583
Number of mitoses[c,b]	1.5 (1.0, 2.0)	2.0 (2.0, 2.0)	2.0 (1.0, 4.0)	2.0 (1.0, 4.0)	0.7862

[a] Statistics listed in the columns are frequency (percent). *p*-Value is based on a Fisher's Exact test.
[b] Each of these 6 parameters were scored for each tumor using a 4-point rating scale, with a score of 1 corresponding to a more differentiated presentation (e.g. abundant keratinization), and a score of 4 corresponding to the least differentiated presentation (e.g. absence of keratinization). A composite score was also calculated for each tumor as the sum of the individual scores in this classification system. The tumor thickness was measured in millimeters microscopically for each tumor, as described by Moore et al.[37]
[c] Statistics listed in the columns are median (minimum, maximum). *p*-Value is based on a Kruskal–Wallis test.

DMP1 and MEPE expression. To our knowledge, this is the first study demonstrating the presence of BSP and DSPP in human OSCC surgical resections. Several reports have indicated the up-regulation of BSP, DSPP, OPN, and DMP1 in cancers of other regions of the body such as lung, breast, uterus, thyroid and colon,[14–28] while MEPE expression so far has been limited to tumors associated with phosphate wasting.[8,28]

The statistically significant correlations between the levels of BSP and OPN on the one hand, and their respective

Table 3 Relationship between DSPP expression levels of T1/T2 tumors and clinical variables

Variable	0	1	2	3	*p*-Value
Sex[a]					0.8669
Male	4 (66.7)	0 (0.0)	9 (75.0)	5 (62.5)	
Female	2 (33.3)	0 (0.0)	3 (25.0)	3 (37.5)	
Race[a]					1.0000
Black	0 (0.0)	0 (0.0)	1 (8.3)	1 (12.5)	
Caucasian	6 (100.0)	0 (0.0)	11 (91.7)	7 (87.5)	
Tumor location[a]					0.0163
FOM	0 (0.0)	0 (0.0)	7 (58.3)	6 (75.0)	
Retromolar pad	0 (0.0)	0 (0.0)	1 (8.3)	0 (0.0)	
Tongue	6 (100.0)	0 (0.0)	4 (33.3)	2 (25.0)	
Histology[a]					0.4216
Poor	0 (0.0)	0 (0.0)	1 (9.1)	0 (0.0)	
Moderate	3 (60.0)	0 (0.0)	4 (36.4)	5 (83.3)	
Well	2 (40.0)	0 (0.0)	6 (54.5)	1 (16.7)	
Metastasis[a]					0.6422
Yes	1 (16.7)	0 (0.0)	5 (41.7)	2 (25.0)	
No	5 (83.3)	0 (0.0)	7 (58.3)	6 (75.0)	
Keratin[a]					0.3517
1	6 (100.0)	0 (0.0)	9 (75.0)	4 (50.0)	
2	0 (0.0)	0 (0.0)	1 (8.3)	3 (37.5)	
3	0 (0.0)	0 (0.0)	1 (8.3)	1 (12.5)	
4	0 (0.0)	0 (0.0)	1 (8.3)	0 (0.0)	
Pattern of invasion[a]					0.9424
1	1 (16.7)	0 (0.0)	3 (25.0)	1 (12.5)	
2	2 (33.3)	0 (0.0)	6 (50.0)	4 (50.0)	
3	1 (16.7)	0 (0.0)	2 (16.7)	2 (25.0)	
4	2 (33.3)	0 (0.0)	1 (8.3)	1 (12.5)	
Stage of invasion[a]					0.7575
1	0 (0.0)	0 (0.0)	3 (25.0)	1 (12.5)	
2	4 (66.7)	0 (0.0)	7 (58.3)	6 (75.0)	
3	2 (33.3)	0 (0.0)	2 (16.7)	1 (12.5)	
Age[b]	61.5 (45.0, 79.0)	—	65.5 (49.0, 86.0)	63.5 (53.0, 81.0)	0.7957
Depth of invasion [b]	1.6 (0.8, 7.0)	—	2.0 (0.5, 6.0)	2.7 (0.6, 3.0)	0.9046
ETOH[b]	0.0 (0.0, 7.0)	—	6.0 (0.0, 11.0)	4.0 (0.0, 6.0)	0.0798
Smoking[b]	42.5 (0.0, 720.0)	—	60.0 (20.0, 240.0)	54.0 (0.0, 120.0)	0.6444
Lymph infiltration[b]	3.0 (2.0, 4.0)	—	2.0 (1.0, 3.0)	2.0 (1.0, 4.0)	0.1626
MMP-2[b]	1.5 (0.0, 3.0)	—	2.0 (1.0, 3.0)	2.5 (0.0, 3.0)	0.4176
MMP-3[b]	1.0 (0.0, 3.0)	—	3.0 (1.0, 3.0)	2.0 (1.0, 3.0)	0.1175
MMP-9[b]	0.0 (0.0, 1.0)	—	0.0 (0.0, 2.0)	0.0 (0.0, 2.0)	0.5448
Nuclear polymorphism[b]	2.0 (1.0, 3.0)	—	1.0 (1.0, 4.0)	2.0 (1.0, 4.0)	0.4024
Number of mitoses[b]	2.0 (1.0, 3.0)	—	1.0 (1.0, 4.0)	2.0 (1.0, 4.0)	0.4499

[a] Statistics listed in the columns are frequency (percent). *p*-Value is based on a Fisher's Exact test.
[b] Statistics listed in the columns are median (minimum, maximum). *p*-Value is based on a Kruskal—Wallis test.

MMP partners, MMP-2 and MMP-3, are not entirely surprising in the light of earlier reports on the expression of these proteases in OSCC. For example Gao and colleagues recently reported the expression of MMP-2 in OSCC tissues.[39] These authors also showed that MMP-2 expression was related to the differentiation status of the tumor cells, presence or absence of regional lymph node metastases, and the stage of OSCC, but not related to the age and gender of patients, or the oral location of the OSCC. The authors therefore suggested that MMP-2 may play important roles in the invasion and metastasis of OSCC.

Mirroring the recent observations in select normal soft tissues,[12,13,29] BSP and OPN were expressed with their known MMP partners, MMP-2 and MMP-3, respectively, in OSCC. Also consistent with earlier reports, some OSCC in the present study expressed various levels of MMP-9 although without

Table 4 Relationship between OPN expression levels of T1/T2 tumors and clinical variables

Variable	0	1	2	3	*p*-Value
Sex[a]					0.7629
Male	4 (66.7)	1 (50.0)	5 (62.5)	8 (80.0)	
Female	2 (33.3)	1 (50.0)	3 (37.5)	2 (20.0)	
Race[a]					0.2831
Black	0 (0.0)	0 (0.0)	2 (25.0)	0 (0.0)	
Caucasian	6 (100.0)	2 (100.0)	6 (75.0)	10 (100.0)	
Tumor location[a]					0.8187
FOM	2 (33.3)	1 (50.0)	4 (50.0)	6 (60.0)	
Retromolar pad	0 (0.0)	0 (0.0)	1 (12.5)	0 (0.0)	
Tongue	4 (66.7)	1 (50.0)	3 (37.5)	4 (40.0)	
Histology[a]					0.1518
Poor	1 (25.0)	0 (0.0)	0 (0.0)	0 (0.0)	
Moderate	3 (75.0)	2 (100.0)	4 (50.0)	3 (37.5)	
Well	0 (0.0)	0 (0.0)	4 (50.0)	5 (62.5)	
Metastasis[a]					0.3245
Yes	2 (33.3)	0 (0.0)	1 (12.5)	5 (50.0)	
No	4 (66.7)	2 (100.0)	7 (87.5)	5 (50.0)	
Keratin[a]					0.3934
1	5 (83.3)	2 (100.0)	5 (62.5)	7 (70.0)	
2	0 (0.0)	0 (0.0)	1 (12.5)	3 (30.0)	
3	0 (0.0)	0 (0.0)	2 (25.0)	0 (0.0)	
4	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)	
Pattern of invasion[a]					0.5599
1	2 (33.3)	0 (0.0)	1 (12.5)	2 (20.0)	
2	1 (16.7)	1 (50.0)	4 (50.0)	6 (60.0)	
3	1 (16.7)	0 (0.0)	2 (25.0)	2 (20.0)	
4	2 (33.3)	1 (50.0)	1 (12.5)	0 (0.0)	
Stage of invasion[a]					0.9293
1	1 (16.7)	0 (0.0)	1 (12.5)	2 (20.0)	
2	4 (66.7)	1 (50.0)	5 (62.5)	7 (70.0)	
3	1(16.7)	1 (50.0)	2 (25.0)	1 (10.0)	
Age[b]	60.5 (45.0, 79.0)	61.5 (54.0, 69.0)	70.0 (57.0, 86.0)	64.5 (49.0, 82.0)	0.2892
Depth of invasion[b]	1.8 (0.8, 7.0)	1.8 (1.6, 2.0)	2.5 (0.5, 4.2)	2.8 (0.5, 6.0)	0.8969
ETOH[b]	0.0 (0.0, 6.0)	6.0 (5.0, 7.0)	5.0 (0.0, 11.0)	5.0 (0.0, 10.0)	0.2939
Smoking[b]	54.0 (40.0, 720.0)	70.0 (20.0, 120.0)	46.0 (0.0, 240.0)	52.5 (0.0, 120.0)	0.9786
Lymph infiltration[b]	2.5 (1.0, 4.0)	3.0 (3.0, 3.0)	2.5 (1.0, 3.0)	2.0 (1.0, 4.0)	0.7110
MMP-2[b]	1.0 (0.0, 3.0)	1.0 (0.0, 2.0)	3.0 (2.0, 3.0)	2.0 (2.0, 3.0)	0.0102
MMP-3[b]	1.0 (0.0, 2.0)	2.0 (1.0, 3.0)	3.0 (2.0, 3.0)	3.0 (1.0, 3.0)	0.0122
MMP-9[b]	0.5 (0.0, 2.0)	0.5 (0.0, 1.0)	0.0 (0.0, 1.0)	0.0 (0.0, 2.0)	0.3247
Nuclear polymorphism[b]	1.0 (1.0, 3.0)	1.5 (1.0, 2.0)	1.5 (1.0, 4.0)	2.5 (1.0, 4.0)	0.2773
Number of mitoses[b]	1.0 (1.0, 2.0)	2.0 (2.0, 2.0)	2.0 (1.0, 4.0)	1.5 (1.0, 3.0)	0.3403

[a] Statistics listed in the columns are frequency (percent). *p*-Value is based on a Fisher's Exact test.
[b] Statistics listed in the columns are median (minimum, maximum). *p*-Value is based on a Kruskal–Wallis test.

concurrent expression of its partner SIBLING, DMP1. This observation re-enforces our earlier conclusion that, while SIBLINGs with known MMP partners always co-express with their MMP partners, sometimes the MMPs are expressed without their SIBLING counterparts.[13] This is not entirely surprising given the widespread nature of MMPs. These instances of SIBLING-MMP partnering in both normal and pathologic tissues suggest that, for at least BSP and OPN, activity may be specific and dictated by the presence and interaction with specific MMP partners. To date, DSPP and MEPE MMP partners (if any) are unknown.

The roles of MMPs in tumor invasive processes are frequently noted[40,41] and the possible roles of SIBLING-MMP complexes in the biology of cancers can be considered a logical extension. For example, a recent report indicated that the *in vitro* invasiveness of some cancer cell lines were enhanced via the formation of a RGD-dependent complex with MMP-2 and $\alpha_v\beta_3$ integrin.[42] Similarly, some insight has

Table 5 Spearman rank correlation coefficients (T1/T2 tumors)

Variable	BSP		DSPP		OPN	
	r_s	*p*-Value	r_s	*p*-Value	r_s	*p*-Value
Age	0.2234	0.2727	0.0377	0.8551	0.1168	0.5698
Depth of invasion	0.0707	0.7313	0.0836	0.6847	0.1222	0.5521
ETOH	0.2216	0.2871	0.1786	0.3930	0.2176	0.2960
Smoking	0.0381	0.8663	0.0381	0.8663	−0.0792	0.7260
Keratin	0.0586	0.7762	0.3774	0.0573	0.0698	0.7348
Lymph infiltration	−0.0467	0.8208	−0.2738	0.1759	−0.1086	0.5974
MMP-2	**0.7245**	**<0.0001**	0.1946	0.3308	0.3736	0.0549
MMP-3	**0.7227**	**<0.0001**	0.1845	0.3569	**0.5594**	**0.0024**
MMP-9	−0.2084	0.2970	0.1418	0.4806	−0.0582	0.7730
Nuclear polymorphism	−0.0865	0.6745	0.1345	0.5123	0.3647	0.0670
Number of mitoses	0.1221	0.5523	−0.0364	0.8599	0.1229	0.5497

recently been provided as to a potential role of the DMP1-MMP-9 pair in cancer progression and metastasis by showing coordinated increase in MMP-9 and DMP1 expression in colon cancer following a cancer (cDNA) array analysis.[28] Additional findings indicate that DMP1 enhances the *in vitro* invasion potential of a specific colon cancer cell line by bridging MMP-9 to integrins and, perhaps, CD44.[43] Increased expression of MMP-9 in colon cancer has also been shown to be associated with metastasis to the liver.[43]

Distant metastasis in OSCC is defined as spread to organs below the clavicle (beyond regional cervical lymph node spread). In contrast to colon cancer where distant metastasis occurs early in the course of disease,[27] distant metastases are rare and typically occur late during the course of OSCC.[2] As a result, OSCC patients often die from locoregional spread rather than classic distant organ metastasis. Thus, although MMP-9 is expressed in some OSCC, lack of expression of its SIBLING partner, DMP1, may indicate a relative handicap in a coordinated DMP1-MMP-9 up-regulation and partnering necessary for initiating the process of distant organ metastasis similar to that observed in colon cancers. Thus, the absence of DMP1 in primary OSCC may account for the rarity of early distant organ metastasis.

Analyses of BSP, DSPP, and OPN expression levels did not show any significant differences with tumor T-stage and most clinical parameters (Tables 2—4). With respect to primary tumor location, analysis of each family member showed that DSPP expression was higher (scores of 2 or 3) for floor of mouth (FOM) and retromolar region lesions than for the tongue. This observation appears counterintuitive given that OSCC of the tongue has the highest rate of regional lymph node metastasis coupled with the most aggressive behavior than those of the FOM and retromolar region. However, we are mindful that the total number of FOM, retromolar region, and tongue lesions are not adequate to provide for a statistical significance test that could be obtained from a cohort involving an adequately large number of OSCC from each location.[41] Thus, further studies will be required to explore any definitive relationship between the level of expression of each of these SIBLINGs, and the oral location of the primary tumor.

Previous reports have indicated the up-regulation of MMP-2 and MMP-9 with moderate- and well-differentiated OSCC.[41,44] Although we observed high SIBLING and cognate MMP expressions in well- and moderately-differentiated tumors, there was complete lack of BSP expression, and low-to-no expression of OPN in poorly differentiated tumors. In contrast, there was sustained up-regulation of DSPP expression in poorly differentiated tumors. Although the significance of these observations is not completely clear at this stage, one plausible explanation is that poorly differentiated OSCC cells turn off BSP and, to a larger extent, OPN genes during the process of ''dedifferentiation.'' One practical consequence of the DSPP observation is that this SIBLING may be a useful serum or saliva marker for OSCC because it may also accurately report on the presence of a significantly undifferentiated tumor in the body.

A recent report also documented the temporal and transient expression of the OPN, BSP, and DMP1 in the cytoplasm of sertoli cells of developing gonads of normal male mice.[45] While the authors proffered no distinct function for BSP and DMP1, they suggested that OPN may be involved in regulating migration, cell process formation, or adhesion during testis development.[45] Given that SIBLINGs are not present in normal human oral mucosa, a possible sequence of events is that there is a temporal onset and intermittent period of expression of the individual SIBLINGs followed by loss of expression of all SIBLINGs at maturity of normal oral mucosa epithelia during oral mucosal development. Subsequent development of oral cancer may turn back the clock to pre-maturation stage mimicking the timing of intermittent expression of specific SIBLINGs during development. Under this scenario, the complete loss of BSP expression and the reduced OPN expression in poorly differentiated tumors may represent a morphogenetic reversal to pre-SIBLING expression stages of oral mucosal development.

Alternatively, loss of BSP and/or OPN may also imply that poorly differentiated OSCC no longer require the activities of these SIBLINGs for their aggressive behavior. Here again, while suggestive, the small number of poorly differentiated OSCC in the present cohort limits the statistical analysis, and therefore, significance of our observation thereby necessitating further studies of a cohort with an adequate number of poorly differentiated tumors.

In summary, our current data suggest that specific members of the SIBLING family, BSP, OPN and DSPP, are not detected in normal oral mucosa but are highly up-regulated

in most OSCC. The production of these SIBLINGs may be independent of the T-stage of OSCC but appear to be dependent on oral location of tumor for DSPP, and the degree of tumor differentiation. We speculate that the production of these SIBLINGs aid local tumor invasion by the activation of the two MMPs: MMP-2 and MMP-3. Furthermore, we speculate that the co-expression of DMP-1 and MMP-9 would have supported distant organ metastases of primary OSCC, an event that is actually rare. If these scenarios are established SIBLING and MMP expression in OSCC may prove to be useful predictors of OSCC potential behavior from locally invasive to distant organ metastatic tumors. In this latter scenario we would predict that primary OSCC with the potential for distant metastasis will express both DMP1 and MMP-9.

Conflict of interest

All authors declare that there is no conflict of interest that could inappropriately influence (bias) this work.

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